A Survey of Synthetic and Natural Phytotoxic Compounds and Phytoalexins as Potential Antimalarial Compounds

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The apicomplexan parasites pathogens such as *Plasmodium* spp. possess an apicoplast, a plastid organelle similar to those of plants. The apicoplast has some essential plant-like metabolic pathways and processes, making these parasites susceptible to inhibitors of these functions. The main objective of this paper is to determine if phytotoxins with plastid target sites are more likely to be good antiplasmodial compounds than are those with other modes of action. The antiplasmodial activities of some compounds with established phytotoxic action were determined *in vitro* on a chloroquine (CQ) sensitive (D6, Sierra Leone) strain of *Plasmodium falciparum*. In this study, we provide *in vitro* activities of almost 50 such compounds, as well as a few phytoalexins against *P. falciparum*. Endothall, anisomycin, and cerulenin had sufficient antiplasmodial action to be considered as new lead antimalarial structures. Some derivatives of fusicoccin possessed markedly improved antiplasmodial action than the parent compound. Our results suggest that phytotoxins with plastid targets may not necessarily be better antiplasmodials than those that act at other molecular sites. The herbicides, phytotoxins and the phytoalexins reported here with significant antiplasmodial activity may be useful probes for identification of new antimalarial drug targets and may also be used as new lead structures for new antiplasmodial drug discovery.

Key words *Plasmodium falciparum*; apicoplast; antiplasmodial; herbicide; phytotoxin; phytoalexin

Discovery of new antimalarial drugs with new modes of action is a primary focus of malaria researchers because resistance is increasing for almost every chemotherapeutic regimen available for the treatment of the disease. Malaria parasites are members of apicomplexa and have a plastid organelle called the apicoplast which performs vital metabolic functions that are important for their complete life cycle.[2)] In plants, plastids are represented in several forms, including chloroplasts, leucoplasts, amyloplasts, and chromoplasts, all containing the same genetic complement (the plastome) within a single plant. Many metabolic processes (*e.g.*, amino acid synthesis) of plant plastids play essential roles in cellular function.[3)]

All plastids originate from cyanobacteria and retain a degree of autonomy, including their own transcription/translation system. There are several pathways and functions present in both the apicoplasts and plant plastids which are fundamentally different from the analogous pathways and functions in humans that might be good targets for antiplasmodial drugs.[4)] Apicoplast function is essential for survival and viability of *Plasmodium* spp., but all of the crucial apicoplast processes are not known.[5)] Determination of apicoplast functions which are necessary for survival of the parasite may provide a source of potential targets for antimalaria drugs.

The cyanobacterial genome consists of about 1500 genes, but plants have only 60—200 plastome-coded proteins, and the plastome of *Plasmodium* spp. encodes 60 proteins.[6,7)] Some of the pathways of photosynthetic plastids remain in the vestigial form in the apicoplast; for example part of photosynthetic electron transport chain.[5)] Most of genes of cyanobacteria were transferred to the nucleus during plastid evolution, and the protein products of some of these nuclear genes are plastid localized, so that plastid function requires a complicated interplay between plastome and genome.[6)] Plastome- or genome-encoded, apicoplast proteins might be selective target sites for *Plasmodium* spp., as mammals do not have this organelle. Comparison of plastomes from plants and apicomplexan parasites may be useful in identification of the functions of the proteins involved in plastid-localized metabolic pathways of *Plasmodium* spp.

Many highly effective herbicides and natural phytotoxins target plastid processes such as chlorophyll biosynthesis, photosynthetic electron flow, chloroplast isoprenoid biosynthesis, and synthesis of amino acids.[8)] Some of these pathways may be common to both plant and apicomplexan plastids. Therefore, phytotoxic compounds with plastid target sties might be effective against the apicomplexa. Others have had similar ideas, but have published results with only a limited number of compounds representing very few molecular target sites.[9)] To test this theory, we have examined the activity of a broad range of both natural and synthetic phytotoxins, some with plastid target sites and others with non-plastid target sites, against *Plasmodium falciparum*. Furthermore, artemisinin, a phytochemical involved in plant defense, has been a very successful antimalarial drug.[10)] So, in this paper, we have also examined antiplasmodial activity of some plant-synthesized, stilbene-based phytoalexins, known to play a role in plant defense.[11)]

MATERIALS AND METHODS

Sources of Test Compounds Cavoxin and cavoxone were purified, respectively, as pale yellow and white needles from the culture filtrates of *Phoma cava* as previously reported, as well as papuline from the culture filtrates of *Pseudomonas syringae* pv. *papulans* but also by esterification of the (*S*)-2-hydroxy-ephenylpropanoic acid.[12,13)] Cycasin was purified from the aqueous extract of hulled seeds of

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Cycas revolute as previously reported.[14]

Fusicoccin (FC) was produced by *F. amygdali* as reported.[15] The crystalline sample of FC obtained as previously reported preserved at -20 °C under dark for about 26 years showed by TLC (silica gel, eluent $CHCl_3$–*iso*-PrOH, 9 : 1) and ^{1}H-NMR analyses the presence of some minor alteration products, that probably are the well known isomer formed by the shift of the acetyl group from the C-3 to C-2 and C-4 of the glucosyl residue, respectively, (*allo*- and *iso*-FC).[16,17] So that the sample was purified by a silica gel column chromatography (eluent $CHCl_3$–*iso*-PrOH, 9 : 1). The corresponding dideacetyl derivative (DAF), isolated in low yield from *F. amygdali* culture filtrates, was also prepared by alkaline hydrolysis of FC according to the procedure previously reported and purified by preparative TLC (silica gel, $CHCl_3$–*iso*-PrOH, 4 : 2).[18] This latter was also used to prepare the deacetylaglycone-FC, through the sugar oxidation followed by a β-elimination reaction as previously reported.[16] The deacetylaglycone-FC was converted by acid catalized reaction with acetone in the corresponding 8,9-isopropylidene derivative (acetonide).[16] The crude sample was purified by preparative TLC (silica gel, $CHCl_3$–*iso*-PrOH, 4 : 2). The purity sample of FC, DAF and acetonide were checked by TLC and ^{1}H-NMR analysis.

The other FC derivatives and analogues, whose purity was ascertained by TLC and ^{1}H-NMR, were prepared starting from FC according to the references listed below: triacetyl-FC, triacetyl-8-oxo-FC, de-*t*-pentenyl-7-*epi*-9-*epi*-tetraacetyl-FC; Ψ-acetonide-FC; isomer of 16-*O*-demethyl-de-*t*-pentenylhexaacetyl-FC; isomer of FC-aglycone; 19-trityl-12-oxo-acetonide.[19—23] The 16-*O*-demethyl-19-deoxydideacetyl-3-*epi*-FC was isolated from *F. amygdali* culture filtrates.[24] The 19-deoxy-12-oxo-acetonide was recently prepared by oxidation with a pyridinium chlorochromate of the acetonide of the 19-deoxydideacetylfusicoccin, a minor metabolite purified from *Fusicoccum amygdali* culture filtrates, according to the procedure previously reported.[25,26] As recently described, the 19-deoxy-12-*epi*-acetonide was prepared by $NaBH_4$ reduction of the corresponding 12-oxoderivative cited above.[25]

Piceatannol and resveratrol were obtained from commercial sources (Calbiochem-Novabiochem Corp., San Diego, CA and Sigma-Aldrich Corp., St. Louis, MO, U.S.A.; respectively). Desoxyrhapontigenin, pinostilbene, pterostilbene, 3-hydroxy-5,4′-dimethoxystilbene and resveratrol trimethylether were synthesized by partial methylation of resveratrol using dimethyl sulfate, following published procedures.[27] The methylether analogs were monitored by thin layer chromatography (TLC) using silica gel plate (Merck Silica gel 60 F_{254}; EMD Chemicals Inc., Gibbstown, NJ, U.S.A.), with hexane : ethyl acetate (8 : 2) as developing solvent. The *Rf* values for resveratrol trimethylether, 3-hydroxy-5,4′-dimethoxystilbene, pterostilbene, pinostibene and desoxyrhapontigenin are 0.80, 0.65, 0.61, 0.28, and 0.20, respectively. The analogs were isolated by preparative layer chromatography using the same solvent for TLC, and their structures were confirmed by ^{1}H-NMR spectroscopy and mass spectrometry.

Technical grade samples of all synthetic herbicides were obtained from Chem Service (West Chester, PA, U.S.A.), except for acifluorfen, glufosinate, and trifluralin obtained from Sigma, beflubutamid obtained from Ube Industries (Ube, Japan), EPTC obtained from Supelco, norflurazon obtained from Sandoz Crop Protection (now Syngenta), and sulcotrione obtained from Zeneca Corp. (now Syngenta).

***In Vitro* Antiplasmodial Bioassay** The antiplasmodial activity of test compounds was determined *in vitro* on a chloroquine (CQ) sensitive (D6, Sierra Leone) strain of *Plasmodium falciparum*. Each test compound was evaluated at eight different concentrations to determine IC_{50} values. The 96-well microtitre plate assay is based on evaluation of the effect of the compounds on growth of asynchronous cultures of *P. falciparum*, determined by the assay of parasite lactate dehydrogenase (pLDH) activity.[28] The appropriate dilutions of the compounds were prepared in DMSO or RPMI-1640 medium (Gibco-BRL) and added to the cultures of *P. falciparum* (2% hematocrit, 2% parasitemia) set up in clear, flat-bottomed, 96-well plates. The plates were placed into the humidified chamber and flushed with a gas mixture of 90% N_2, 5% CO_2 and 5% O_2. The cultures were incubated at 37 °C for 72 h. Growth of the parasite in each well was determined by pLDH assay using Malstat® reagent.[28] The medium and RBC controls were also set-up in each plate.

Ortholog Analysis The names of *Arabidopsis thaliana*'s target proteins were confirmed with the Enzyme Database Brenda (http://www.brenda.uni-koeln.de/index.php4).[29] The OrthoMCL DB (http://orthomcl.cbil.upenn.edu/cgi-bin/OrthoMclWeb.cgi) was searched for the *Plasmodium falciparum* orthologs.[30] Other information about *P. falciparum* orthologs were found in PlasmoDB (http://www.plasmodb.org/plasmo/home.jsp).

RESULTS

We searched the *P. falciparum* data base (PlasmoDB) for orthologs of known genes encoding protein targets for some of the phytotoxins that we evaluated. Known plant targets, mainly enzymes, were analyzed by OrthoMCL DB and Enzyme Database Brenda. The orthologs for ten target proteins were discovered in the *P. falciparum* genome (Tables 1, 2).

Most of synthetic herbicides possessed moderate antiplasmodial activity (Table 1). Endothall, showed promising *in vitro* antiplasmodial activity with an IC_{50} of 7.8 ± 0.4 μM (Table 1). Atrazine was not very active in our bioassay, even though other triazines have been reported to have significant activity.[31]

Most of the natural phytotoxins were active, with anisomycin, cavoxone, and cerulenin being the most active (Table 2). Anisomycin is active against translation of nuclear-coded genes, by binding to the ribosome.[32] Cerulenin inhibits fatty acid synthesis in plants. The mode of action of the cavoxone is unknown. Fusicoccin blocks 14-3-3 proteins that are at the cross-point of a huge array of signaling and regulatory pathways.[33] Fusicoccin though showed only moderate antiplasmodial activity *in vitro*, while some derivatives of fusicoccin were significantly active against *P. falciparum in vitro*, especially isomer of 16-*O*-demethyl-de-*t*-pentenyl-hexaacetyl-FC, 19-trityl-12-oxo-acetonide and 19-deoxy-12-oxo-acetonide (1 ± 0, 2.0 ± 0.6 and 3.0 ± 1.2 μM, respectively) (Fig. 1, Table 3). Phytoalexins are antimicrobial secondary metabolites, which are produced by plants in defense against pathogenic microbes.[34] Some stilbene-based phytoalexins

Table 1. Activity of Herbicides with Different Molecular Target Sites in Plants against *Plasmodium falciparum in Vitro*

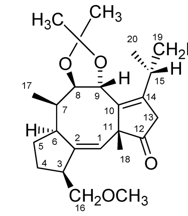
Herbicide name	Molecular target site or mode of action	IC_{50} (μM)[a]
Acetochlor	Inhibits synthesis of very long chain fatty acid synthesis	97±4.2
Acifluorfen[c]	Protoporphyrinogen oxidase[b]	400±0
Aminotriazole	Inhibition of synthesis of membrane lipids and chloroplastic terpenoid derivates	>1000
Asulam	Dihydropteroate synthase[b]	>1000
Atrazine	D1 of Photosystem II	995±7
Beflubuamid	Phytoene desaturase	780±28
Bentazon	D1 of Photosystem II	350±0
Clomazone	DXP synthase	120±0
Dichlobenil	Cellulose synthesis	>1000
Diclofop methyl	Lipid synthesis (ACCase) inhibitor[b]	>1000
Diuron	D1 of Photosystem II	>1000
Endothall	Protein phosphatase[b]	7.8±0.4
EPTC	Fatty acid synthesis	895±7
Fluridone	Phytoene desaturase	120±0
Glufosinate	Glutamine synthetase[b]	730±183
Glyphosate	EPSP synthase[b]	455±63
Imazethapyr	Inhibitor of branched chain amino acid synthesis	>1000
Metsulfuron	Acetolactate synthase	500±0
Norflurazon	Phytoene desaturase	1000±212
Sulcotrione	*p*-Hydroxyphenylpyruvate dioxygenase	790±14
Trifluralin	Tubulin[b]	560±84
Chloroquine		0.014±0.004

a) Values are mean±S.D. of three different observations. *b*) Ortholog gene found in *Plasmodium falciparum* genome. *c*) Herbicides with a plastid target site are in bold italics.

Table 2. Activity of Natural Phytotoxins with Different Molecular Target Sites in Plants against *Plasmodium falciparum in Vitro*

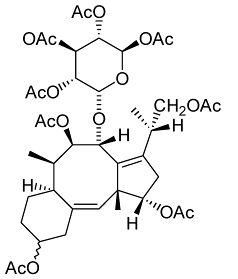
Phytotoxin	Source/target site	IC_{50} (μM)[a]
Actinonin[c]	*Bacillus stearothermophilus*/ peptide deformylase[b]	28±3
Anisomycin	*Streptomyces griseolus*/peptidyl transferase center in 28s RNA[b]	4.6±4
Cavoxin	*Phoma cava*	515±20
Cavoxone	*Phoma cava*	14±4
Cerulenin	*Cephalosporium caerulens*/fatty acid synthesis-β-ketoacyl-ACP synthase[b]	10.1±3.3
Coronatine	*Pseudomonas syringae*/inhibits processes affected by jasmonic acid	305±77
Cycasin	*Cycas* spp.	780±210
Papuline	*Pseudomonas* s. pv. *papulans*	650±285

a) Values are mean±S.D. of three different observations. *b*) Ortholog gene found in *Plasmodium falciparum* genome. *c*) Herbicides with a plastid target site are in bold italics.



19-Deoxy-12-oxo-acetonide R=H

19-Trityl-12-oxo-acetonide R= OC(Ph)$_3$



Isomer of 16-*O*-demethyl -de-*t*-pentenylhexaacetyl-FC

Fig. 1. Structures of the Most Active Compounds in Table 3

Table 3. Activity of Natural Phytotoxins and Derivatives of Fusicoccin against *Plasmodium falciparum in Vitro*

Phytotoxin	Source	IC_{50} (μM)[a]
Fusicoccin (FC)	*Fusicoccum amygdali*	125±00
Dideacetyl-FC (DAF)	*Fusicoccum amygdali*	78±38
Triacetyl-8-oxo-FC	Synthesized from FC	88±30
8,9-Isopropyliden aglycone of FC (acetonide)	Synthesized from FC	313±90
Ψ-Acetonide-FC	Synthesized from FC	55±4
Isomer of 16-*O*-demethyl-de-*t*-pentenylhexaacetyl-FC	Synthesized from FC	1.0±0.0
19-Deoxy-12-oxo-acetonide	Synthesized from 19-deoxy-FC	3.0±1.2
19-Deoxy-12-*epi*-acetonide	Synthesized from 19-deoxy-FC	215±28
19-Trityl-12-oxo-acetonide	Synthesized from FC	2.0±0.6
16-*O*-demethyl-19-deoxy dideacetyl-3-*epi*-FC	*Fusicoccum amygdali*	65±5
Isomer of FC-aglycone	Synthesized from FC	>1000
Triacetyl FC	Synthesized from FC	50±16
De-*t*-pentenyl-7-*epi*-9-*epi*-tetraacetyl-FC	Synthesized from FC	6.0±3.6

a) Values are mean±S.D. of three different observations.

Table 4. Activity of Stilbene-Based Phytoalexins against *Plasmodium falciparum in Vitro*

Phytoalexins	IC_{50} (μM)[a]
Desoxyrhapontigenin	75±20
Piceatannol	120±48
Pinostilbene	66±27
3-Hydroxy-5,4′-dimethoxystilbene	70±16
Pterostilbene	70±16
Resveratrol	60±8.1
Resveratrol trimethylether	94±45

a) Values are mean±S.D. of three different observations.

also showed moderate antiplasmodial activity *in vitro*, with IC_{50} values ranging from 66 to 120 μM (Table 4).

DISCUSSION

The vital roles of the apicoplast and its plant-like metabolic pathways in the survival of apicomplexan parasites have been demonstrated.[5] This has provided strong rationale for searching the *P. falciparum* genome data-base for orthologs of plant genes, which have been shown as the targets for action of phytotoxins and also evaluation of their action against *P. falciparum*. Only ten orthologs of plants genes encoding known phytotoxin targets of phytotoxins used in this study were discovered in the *P. falciparum* genome (Tables 1, 2). More detailed analyses of these orthologs might help to determine molecular characteristics of protein target sites, which may help to modify structures of the compounds known to target these proteins.

Endothall, a dicarboxylic acid herbicide, had the best antiplasmodial activity among the commercial herbicides tested. This compound inhibits protein phosphatases (PP1, PP2A) from mammals and plants, but its mode of action as a herbicide is still unclear.[35,36] Recent studies have shown that the function of enzyme PP1 is essential for *P. falciparum* during release of mature merozoits from erythrocytes.[37] Protein phosphatases play important roles in signal transduction pathways. Protein phosphatases and kinases maintain a delicate balance between phosphorylated and dephosphorylated forms of certain proteins. In this manner they regulate gene expression, cellular proliferation, cell differentiation and apoptosis.[38] Likewise, PP2A is involved in tumor development. Many viral proteins exist which act as modulators of activity and specificity of protein phosphatases.[38] In this way viruses are able to control the situation in the host cell. Therefore, protein phosphatases seem to be interesting antimalarial drug targets due to their highly conserved amino sequence in different species.[38] The *P. falciparum* PP2A (PfPP2A) sequence has high homology to PP2A from other species. That is apparently why the native PfPP2A have very similar biochemical properties to other PP2A, such as high sensitivity to okadaic acid and Mg^{+2}- and Mn^{+2}-dependent activity.[39]

In our studies, atrazine, clomazone, diclofop-methyl, glyphosate, and trifluralin had relatively little activity against *P. falciparum*. In some previous studies, these compounds or closely related analogs have been reported to be of interest as antiplasmodials.

Clomazone was moderately active in our assay. This compound must be bioactivated to form 5-hydroxyclomazone in order to inhibit the non-mevalonic isoprenoid pathway of the plastid.[40] 5-Hydroxyclomazone inhibits deoxyxylulose 5-phosphate synthase (DXP synthase), the first committed step to the non-mevalonic pathway for isoprenoid biosynthesis. Earlier, Mueller C. *et al.*[41] proposed that clomazone had a mechanism of action similar to the natural product fosmidomycin. However, fosmidomycin inhibits DXP reductoisomerase (DXR), the enzyme of the non-mevalonic isoprenoid pathway following DXP synthase. Fosimidomycin is an excellent antiplasmodial agent with good antimalarial activity *in vivo*.[42] Although, the non-mevalonic isoprenoid pathway has been touted as an excellent antimalarial drug target site, most of the efforts have been concentrated on DXR inhibitors, with relatively little research on DXP synthase inhibitors.[43,44]

The acetyl CoA carboxylase (ACCase) inhibitor diclofop methyl was not effective in our study, but this may have been due to the inability of *P. falciparum* to demethylate it to the active form, as plants do. However, Bork S. *et al.*[45] reported clodinafop-propargyl, another aryloxyphenoxypropionate herbicide that must be bioactivated to its acid form, is active against *Plasmodium* spp. and *Toxoplasma* sp. Others have reported several herbicidal aryloxyphenoxypropionate herbicides including diclofop, to have moderate activity against *Toxoplasma gondii*.[46] The herbicidal cyclohexanedione ACCase inhibitors such as sethoxydim were inactive in their study.

Glyphosate, an inhibitor of the shikimate pathway, almost completely inhibited the growth of the asexual erythrocytic forms of *P. falciparum* at about 1 mM.[47] The inhibition could be reversed by folate and *p*-aminobenozoate, indicating that the shikimate pathway is required for folate synthesis in *P. falciparum*. Based on these results, Roberts C. W. *et al.*[48] suggested that glyphosate or other inhibitors of the shikimate pathway might be good antimalarials. Considering the high IC_{50} of glyphosate, this compound has not been further studied or developed for treatment for malaria. Furthermore, extensive searches for other good inhibitors of EPSP synthase, the target enzyme of glyphosate, have been unsuccessful, probably because glyphosate is a transition state analog with little or no structural diversity possible for inhibition of EPSP synthase.

There has been significant interest in triazines as antimalarials.[31] The triazine compounds that have been reported to have good activity against *P. falciparum* apparently act as antifolates.[49] Triazine herbicides, such as atrazine, that inhibit photosynthetic electron transport in plants are apparently not effective in this capacity. Antifolates are of interest as antiplasmodials, however, we have not found any other report of asulam, a potent inhibitor of folate in plants, as an antiplasmodial.[50]

The tubulin-binding herbicide trifluralin had only weak *in vitro* antiplasmodial activity in our assays. An earlier study found that trifluralin and other related dinitroanilines herbicides were all active against *P. berghei in vitro* at, or close to, submicromolar concentrations.[51] But, these researchers found that neither trifluralin nor oryazlin were active *in vivo* in a *P. berghei*–rat malarial model even at the maximum doses tolerated by rats. Trifluralin binds α-tubulin, preventing assembly of the microtubule at the assembly end.[52] *P. falciparum* is much more sensitive to trifluralin than are humans.[53] *Toxoplasma gondii* is highly sensitive to trifluralin and other dinitroaniline mitotic inhibitors.[54]

Although 15 out of 26 herbicides and phytotoxins tested have their target site in the chloroplast, they had only negligible or moderate action against the malaria parasite *in vitro*. The apicoplast has lost most of its chloroplast functions.[5] However, understanding their mechanism of antiplasmodial action may provide potential targets for antimalarial drug discovery. Determination of whether the herbicides and phytotoxins with significant antimalarial action act through the listed orthologous targets or through other mechanisms would be important.

The antiplasmodial activity of fusicoccin (FC), a phytotoxin synthesized by the plant pathogenic fungus *Fusicoccum amygdale*, and its semisynthetic derivates was of interest. FC had only mild antiplasmodial activity but some of its semisynthetic derivatives were much more potent antiplasmodial compounds. FC causes membrane hyperpolarization and change of ionic gradients in leaf cells.[55] It acts as a 14-3-3 protein-mediated activator of the plasma membrane H(+)-ATPase and the biochemical, and physiological changes induced in the cell by this toxin have been mainly ascribed to the increased rate of proton extrusion by this pump leading to external acidification and cell hyperpolarization.

Our results show that both synthetic and natural phytotoxins and phytoalexins have activity against *Plasmodium falciparum*. However, we still do not know enough about the mode of action of some of these compounds to make any generalizations about whether plastids provide a source of good, selective targets for antiplasmodials. The knowledge of molecular target sites in plants of many phytotoxins and identification of orthologs of the genes encoding these sites in *P. falciparum* should be useful in identification of new antimalarial drug targets. This information could also provide the basis for analysis of structure–activity relationships of antiplasmodial activity if these phytotoxins and their analogues. The herbicides, natural phytotoxins, and the phytoalexins reported here with significant antiplasmodial activity may be useful as new lead structures.

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